Citrate transporters of Bacillus subtilis

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SUMMARY

Living cells are surrounded by a membrane that shields the content of the cell (the cytoplasm) from the outside. This membrane, the cytoplasmic membrane, has several functions, of which energy transduction is a very important one. Energy transduction occurs via the formation of concentration gradients of ions across the membrane which can be created and maintained by several energy requiring systems. For example, energy derived from sugar metabolism or from light can be used by specialized systems to pump protons out the cell. This results in a lower concentration of protons inside of the cell relative to outside. Because protons are positively charged this difference in proton concentration results also in a difference in charge (negative inside relative to outside). These gradients exert an inward directed force on the protons located at the outside. The force is termed the proton motive force (pmf). Energy stored in the pmf can be used for several energy requiring processes one of which is transport of solutes across the cytoplasmic membrane.

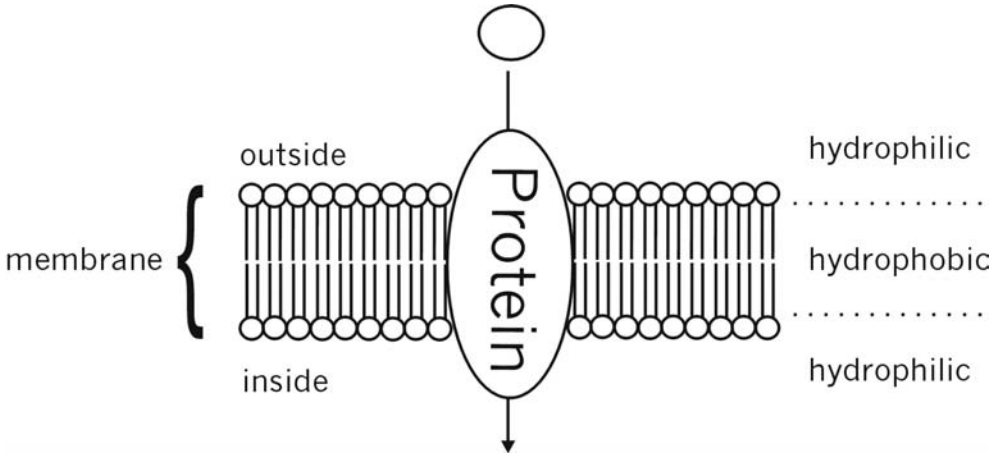

Figure 1. Schematic representation of the cytoplasmic membrane with an embedded transport protein. The headgroups of the phospholipids are indicated by circles while the tailgroups are drawn as lines. The separation between hydrophobic and hydrophilic regions of the system is indicated on the right-hand side of the figure. In this case, inward directed transport of a single solute is shown.

The cytoplasmic membrane consists of proteins and (phospho)lipids. The phospholipids have headgroups that are soluble in water (hydrophilic), and usually two tailgroups that are not (hydrophobic). Because of these characteristics, phospholipids assemble into a bilayer in which the headgroups are directed outward, to the waterphase, while the tails are directed towards each other (Figure 1). In the space between the headgroups no water is present and solutes that are soluble in water have great difficulty passing the membrane. To transport water-

soluble substrates across the membrane, cells contain specialized transporter proteins. Proteins consist of a chain of amino acids. Many proteins are enzymes that perform work in the cell. All processes in the living cell are catalyzed by specialized proteins, as is the case for the transport of water-soluble substances across the cytoplasmic membrane. Transport proteins are embedded in the membrane (Figure 1), and catalyze the transport of a solute from one side of the membrane to the other.

One group of transport proteins is the secondary transporters that use the pmf to drive transport. This group of proteins is the subject of the research presented in this thesis. The objective was to investigate the application of secondary citrate transporters to remove heavy metals from aqueous solutions. Two secondary citrate transporters of the soil bacterium *Bacillus subtilis*, CitM and CitH, recognize citrate only when it is complexed to divalent metals. CitM recognizes citrate bound to magnesium, manganese, cobalt, nickel and zinc, while CitH recognizes citrate bound to calcium, barium and strontium (Chapter 2). CitM activity is interesting for biotechnological reasons. The heavy metals nickel, zinc and cobalt are toxic and should not be released in the environment. It was investigated whether CitM activity could be used to remove these heavy metals. The idea was to bind the heavy metals in solution to citrate and accumulate these complexes in bacterial cells. Citrate will than be metabolized within the cell so that the metal will accumulate.

Functional characterization of proteins is usually achieved with molecular biological techniques such as genetic manipulation. The gene encoding a protein of interest can be placed in a bacterial cell and subsequently induced to produce the protein. By comparing the properties of cells producing the protein with non-producing cells information can be obtained about the function of the protein. In this thesis we used this technique to elucidate the function of three citrate transport proteins. The genes encoding these proteins are taken from *B. subtilis* and placed in *Escherichia coli*. *Escherichia coli* was used as a host because this bacterium does not possess a citrate transporter of its own. Thus, the accumulation of citrate in *E. coli* represents the activity of the *B. subtilis* protein. Accordingly, it was shown that CitM and CitH of *B. subtilis* catalyze the transport of citrate in complex with divalent metals, and that the entire complex is transported into the cell (Chapter 2). Because the application of genetically modified organisms is not generally accepted, the application of naturally occurring *B. subtilis* to remove the metals was investigated. It was shown that the activity of CitM in *B. subtilis* does indeed result in increased accumulation of heavy metals in the cell (Chapter 3). However, since heavy metals are toxic, they kill the bacterium. To protect itself, the bacterium changed CitM in such a way that the protein is no longer active and metal is no longer accumulated.

The third citrate transport protein that was studied, CimH, that recognizes free citrate, but not citrate in complex with metals like CitM and CitH (Chapter 4). Besides citrate also L-malate is recognized and transported by CimH. The activity of CimH could potentially interfere with a possible application of CitM or CitH because transport of free citrate could lower the citrate concentration and thus the

amount of metals that is complexed and accumulated. Fortunately, it was shown that CimH is not present when CitM is in *B. subtilis*.

An important question within the research field of secondary transporters, is how these proteins use the pmf to drive transport of a solute across the membrane. To answer this question it is important to get besides functional also structural information on the transport proteins. Detailed structural information of proteins can be obtained using biochemical approaches. A very powerful and widely used method is the insertion of single cysteine residues at defined positions within the protein. Using chemicals that specifically react with these cysteine residues, information on the position and surroundings of the cysteine can be obtained. Questions can be answered such as: is the residue in contact with water?; Is the residue located on the inside or outside of the cell?; Is the residue that has been replaced by the cysteine important for the function of the protein?; Insertion of two cysteines at different positions in the protein allows determination of the distance between these two positions.

CimH belongs to a group of proteins, of which many have been studied in our laboratory. We investigated the structure of CimH by placing single cysteine residues in defined places within the protein. We have elucidated the function of one residue and part of the structure of the protein. A cytoplasmic (internal) loop of the protein is accessible form the exterior of the cell. Either this loop protrudes to the outside, or a water filled pathway exists that contains these residues (Figure 2). Further study on this loop will show which of the two models is correct.

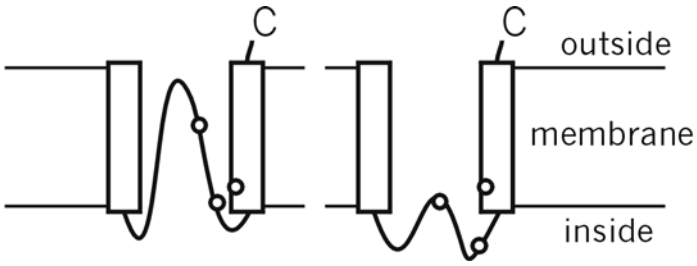

Figure 2. Two models representing the structure of the last cytoplasmic loop of CimH. Circles indicate the positions that have been replaced by cysteine residues, C indicates the C-terminus of the protein. The model on the left-hand side shows the loop protruding to the outside. On the right-hand side the loop is depicted as part of a water filled pathway within the protein.

An overview of all transport protein for citrate or citrate related compounds in *B. subtilis* clearly shows that *B. subtilis* is a versatile organism. Possibly because *B. subtilis* lives in the soil, where it might endure various conditions, the bacterium possesses an array of transport proteins for citrate or citrate related compounds. Many of these proteins have been studied and it appears that different transport proteins catalyze transport of the same compounds. *Bacillus subtilis* is thus able to utilize an array of transport proteins to accumulate the different substrates that it might encounter under different conditions.